A Novel *lux* Operon in the Cryptically Bioluminescent Fish Pathogen *Vibrio salmonicida* Is Associated with Virulence[▽]

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The cold-water-fish pathogen *Vibrio salmonicida* expresses a functional bacterial luciferase but produces insufficient levels of its aliphatic-aldehyde substrate to be detectably luminous in culture. Our goals were to (i) better explain this cryptic bioluminescence phenotype through molecular characterization of the *lux* operon and (ii) test whether the bioluminescence gene cluster is associated with virulence. Cloning and sequencing of the *V. salmonicida lux* operon revealed that homologs of all of the genes required for luminescence are present: *luxAB* (luciferase) and *luxCDE* (aliphatic-aldehyde synthesis). The arrangement and sequence of these structural *lux* genes are conserved compared to those in related species of luminous bacteria. However, *V. salmonicida* strains have a novel arrangement and number of homologs of the *luxR* and *luxI* quorum-sensing regulatory genes. Reverse transcriptase PCR analysis suggests that this novel arrangement of quorum-sensing genes generates antisense transcripts that may be responsible for the reduced production of bioluminescence. In addition, infection with a strain in which the *luxA* gene was mutated resulted in a marked delay in mortality among Atlantic salmon relative to infection with the wild-type parent in single-strain challenge experiments. In mixed-strain competition between the *luxA* mutant and the wild type, the mutant was attenuated up to 50-fold. It remains unclear whether the attenuation results from a direct loss of luciferase or a polar disturbance elsewhere in the *lux* operon. Nevertheless, these findings document for the first time an association between a mutation in a structural *lux* gene and virulence, as well as provide a new molecular system to study *Vibrio* pathogenesis in a natural host.

Marine bioluminescent bacteria have been the subjects of considerable interest because of the biochemistry that drives light production and their ability to initiate specific, long-term cooperative symbioses with many species of squids and fishes (20, 35, 45, 51). Less is known about bioluminescence in species of bacteria that have the capacity to produce light yet are found in pathogenic associations with animal hosts (32, 33, 38). It has always been of interest to know whether luminescence plays a role in the biology of such pathogens, either to colonize the hosts or to grow in environmental niches. However, attempts to address such questions were limited because a model system in which to study the relationship between bioluminescence and pathogenesis was not available.

In the five previously characterized species of luminous bacteria (*Vibrio fischeri*, *Vibrio harveyi*, *Photobacterium leiognathi*, *Photobacterium phosphoreum*, and *Photorhabdus luminescens*), the six structural genes for bioluminescence are contained within a locus termed the *lux* operon. With the exception of a duplication of *luxB* (designated *luxF*) in one species, these genes are arranged in the order *luxCDABEG* (1, 9, 16, 28). *luxA* and *luxB*, respectively, encode the alpha and beta subunits of luciferase, the enzyme responsible for luminescence. *luxC*,

luxD, and *luxE* each encode an enzyme required for the synthesis of an aliphatic-aldehyde substrate. *luxG* is not essential for luminescence but is believed to increase the capacity of the cell to synthesize flavin mononucleotide (FMN) (42). In the luminescence reaction, luciferase converts this aliphatic-aldehyde substrate, oxygen, and reduced FMN ($FMNH_2$) into the corresponding aliphatic acid, water, and FMN, with the concomitant production of light (19, 28). In the absence of the aldehyde substrate, luciferase catalyzes a reaction that yields no light and produces oxygen radicals rather than water (15, 18).

Bacteria that carry the genes for luciferase yet do not produce a detectable level of light in culture have been referred to as cryptically bioluminescent (13), and this phenotype may be quite widespread in the environment (14, 33, 34). Cryptic bioluminescence has been best characterized with the psychrophilic fish pathogen *Vibrio salmonicida* (13), the only bacterium known to cause vibriosis in cold-water, farmed Atlantic salmon (*Salmo salar* L.), as well as rainbow trout and cod (10, 11).

Cultures of *V. salmonicida* become visibly luminous only upon the addition of aliphatic aldehyde (an aldehyde group attached to a linear saturated carbon chain) and induce the synthesis of luciferase 10-fold per cell as they approach stationary phase (13). Similarly, when exposed to *N*-3-oxo-hexanoyl homoserine lactone, the signal molecule that is in part responsible for quorum sensing and the induction of luminescence in *V. fischeri* (30, 32), *V. salmonicida* induces luciferase production 100-fold. Thus, the regulation of the luciferase in *V. salmonicida*, like that in its close congener *V. fischeri*

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference
Bacterial strains		
NCMB 2262	*V. salmonicida* type strain isolated from diseased salmon	8
EN3	*luxA* insertion mutant containing pEN124 chromosomally integrated into NCMB 2262 (Cam[r] Kan[r])	This study
EN4	*luxA* insertion mutant derived from EN3 (Kan[r])	This study
Plasmids		
pEVS79	Derivative of pBS SK+ (Stratagene Inc.) (*mob* site; ColE1 ori)	40
pEVS104	Helper plasmid with *tra* and *trb* genes (Cam[r])	40
pVO8	Vector plasmid with pACYC184 ori (Cam[r] Erm[r])	47
pKV17	Derivative of pHV200 (16); 7.9-kbp SalI fragment encoding the *V. fischeri* ES114 *lux* region (Δ*luxA*)	45
pEN114	10.8-kbp SalI fragment with a portion of the *V. salmonicida lux* operon ligated into pEVS79	This study
pEN115	*V. salmonicida luxAB* PCR product ligated into pCR2.1 (Invitrogen Inc.)	This study
pEN123	*V. salmonicida luxAB* PCR product ligated into pEVS79	This study
pEN124	pEN123 with a transposon (Kan[r]) inserted 987 bp downstream of the start of the *V. salmonicida luxA* ORF	This study
pEN133	SalI fragment with *V. salmonicida lux* sequence 5′ to *luxC* ligated into pEVS79	This study
pEN134	BamHI fragment from pEN114 (*luxDABEG*) ligated into pV08	This study
pEN135	*V. fischeri* ES114 *luxCDBEG* (Δ*luxA*) from pHV200 in frame with the *lacZ* promoter on pVO8	This study

[a] Cam[r], chloramphenicol resistance; Erm[r], erythromycin resistance; Kan[r], kanamycin resistance.

(50), appears to be under the control of a quorum-sensing autoinducer mechanism. Despite these similarities, quorum-sensing regulation of the *lux* operon in *V. fischeri* strain MJ1 is responsible for a 10,000-fold increase in light production per cell (31), which is substantially greater than the 10-fold increase in *V. salmonicida*. Further, unlike that of *V. salmonicida*, the luciferase reaction of *V. fischeri* MJ1 is not limited by the absence of aliphatic aldehyde (31).

To examine the genetic basis for its aliphatic-aldehyde deficiency and low levels of autoinduction, the luminescence gene cluster of *V. salmonicida* was cloned and sequenced. The arrangement and sequence of the structural *lux* genes are conserved compared to those in related species of luminous bacteria; however, *V. salmonicida* has both a novel arrangement and a different number of homologs of the *luxR* and *luxI* quorum-sensing regulatory genes. Transcriptional analysis suggested that this novel arrangement generates antisense transcripts that may be responsible for the reduced production of bioluminescence. Further, mutagenesis of *V. salmonicida luxA* resulted in marked attenuation of virulence of the mutant relative to that of the wild type in both single- and mixed-strain animal challenge experiments.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* DH5α, grown in Luria-Bertani (LB) medium (37) at 37°C, was the host for plasmids with ColE1 or pACYC184 origins of replication. When added to LB medium for the selection of *E. coli* cells carrying a plasmid, ampicillin, chloramphenicol (Cam), and kanamycin (Kan) were used at concentrations of 100, 30, and 50 μg/ml, respectively. *V. salmonicida* strains were originally isolated from diseased Atlantic salmon (8, 39). Unless indicated otherwise, the principal strain used in this study was *V. salmonicida* NCMB 2262[T]. *V. salmonicida* cultures were grown at 15°C with shaking at 150 rpm for 2 to 3 days in a complex broth (SWT) that contained 10 g of tryptone, 3 g of yeast extract, and 3 ml of glycerol per liter of 70% seawater (2). SWT blood agar contained, per liter of SWT broth, 15 g of agar and 50 ml of Alsevers sheep blood (Colorado Serum Co., Denver, CO). When added to SWT for the selection of *V. salmonicida* cells carrying a plasmid, Cam and Kan were used at 2 and 150 μg/ml, respectively.

Triparental mating for *V. salmonicida*. We developed a version of the triparental mating procedure described by Valla et al. (44), adjusted for the differences in the optimal growth temperatures of *E. coli* and *V. salmonicida* (8). The transfer of plasmids to *V. salmonicida* was performed using pEVS104 as a helper

plasmid (40) contained in *E. coli* DH5α. *E. coli* and *V. salmonicida* strains were grown to an optical density (OD) at 600 nm of between 0.5 and 0.8 in LB (37°C) and SWT (15°C) broths, respectively. The cells in 1 ml of each culture were pelleted, washed three times in chilled (4°C) SWT, and resuspended in 5 μl of chilled SWT. The resuspended cells were combined, spotted onto a chilled SWT plate, and placed in a 23°C incubator for 6 h. The plate was subsequently incubated at 15°C for another 12 h. The resulting confluent growth of cells was scraped off the plate, resuspended in 1 ml of chilled SWT broth, and incubated with shaking at 150 rpm for 12 h at 15°C. Following the incubation, the suspension was plated onto antibiotic-containing SWT blood agar plates. After 10 days of growth, colonies of *V. salmonicida* transconjugates were streaked for purification.

Molecular manipulation of the *lux* region. (i) Cloning and sequencing of the *lux* gene cluster. Using standard PCR methods with consensus primers for the *luxAB* region (forward, O-LUXDFP2 [5′-CATGTCATTCGCTA-3′], and reverse, [O-LUXDRP1 5′-AGATAAGATCATCA-3′]), we generated a PCR product that was cloned into the TA cloning vector (Invitrogen, Carlsbad, CA) to make the *luxAB* plasmid pEN115 (Table 1). A Southern probe analysis based on the internal *luxAB* sequence in pEN115 was used to screen a library of SalI genomic fragments of *V. salmonicida* cloned into the vector pEVS79 for *luxAB*-positive clones. One such clone, pEN114, was isolated and sequenced. Because pEN114 lacked the region upstream of *luxC*, we subcloned the *luxAB* fragment from pEN114 into pEVS79 to make pEN123 and marked *luxAB* by using the in vitro EZ::TN <KAN-2> insertion kit (Epicenter Technologies Inc., Madison, WI) to generate pEN124. pEN124 contained a transposon (Kan[r]) insertion near the middle of the *luxAB* fragment (886 bp into the *luxA* open reading frame [ORF]); this transposon has transcriptional terminators at each end. The marked copy of *luxAB* was introduced into the genome of *V. salmonicida* by triparental mating, and the single recombinant, EN3, was selected by sequentially patching colonies onto SWT-Kan, SWT-Cam, and nonselective SWT blood plates. Genomic DNA from EN3 was purified; digested with SacI, which cuts upstream of *luxC*; and ligated into the SacI site in pEVS79. A Kan-resistant clone harboring a pEVS79 derivative containing the marked copy of *luxAB* was found to contain a small portion of the genomic sequence upstream of *luxC*. Primers were designed from this upstream sequence and used to rescreen the *V. salmonicida* SalI library by PCR, resulting in the identification of the plasmid pEN133. The plasmid pEN133 was found to contain a large region immediately upstream of *luxC*, adjacent to the SalI fragment cloned into pEN114. pEN133 was sequenced by standard methods.

(ii) Screening for a double-recombinant *luxA* mutant. Strain EN3 (Cam and Kan resistant) was grown without Cam selection in SWT. Approximately 10,000 colonies from this culture were screened (Cam sensitive, Kan resistant) for the loss of the integrated pEN124 plasmid by double recombination. Subsequent clones were screened by PCR for the integration of the Kan[r] marker. The resulting clone, EN4, was examined by sequencing and Southern blot analysis to confirm that a single integration of the Kan resistance marker had occurred in *luxA*.

(iii) Complementing the *luxA* mutation. pEN114 was digested with BamHI, and the *luxDABEG* fragment was cloned into the BamHI site of pV08. The
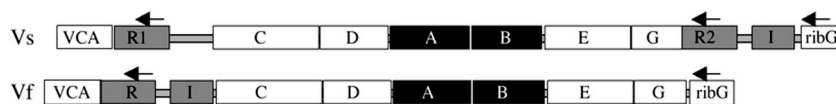
FIG. 1. Organization of bacterial *lux* genes in *V. salmonicida* (Vs) and *V. fischeri* (Vf). VCA represents homologs of the genes for the *V. cholerae* VCA0181 protein (21) and the *V. fischeri* VFA0926 protein (36). *ribG* is a riboflavin synthesis gene (24). Unless indicated otherwise by an arrow, ORFs are predicted to be transcribed from left to right. Black and gray highlighting denotes ORFs corresponding to luciferase genes and *lux* regulatory genes, respectively (27, 29).

resulting plasmid (pEN134) and the parent vector were separately moved into the *luxA* mutant EN4 and the wild-type parent by triparental mating.

DNA sequence analysis of *lux* regions. Stem-loop structures in *lux* gene clusters of several bacterial species were identified using DNA Strider V1.2. The Gibbs free-energy (ΔG) value for each stem-loop was calculated in units of kilocalories per mole by using the program Mfold (GCG, Madison, WI). ΔG values were calculated for the optimal growth temperatures for the following bacterial species: *V. salmonicida* (15°C), *V. fischeri* MJ1 (30°C), *V. harveyi* (35°C), and *P. leiognathi* (35°C).

PCR analysis of the *lux* region arrangement in five *V. salmonicida* strains. PCR was used to putatively identify and map the *luxR1*, *luxR2*, and *luxI* regions (Fig. 1) of *V. salmonicida* strains isolated from independent sources (Table 1). PCRs were performed with genomic DNA by using the primer pairs O-EN11 (5′-GC CAGATCAAATGTTTGCTG-3′) and O-EN20 (5′-GTCACTTGGCTACCGC TCG-3′); O-EN26 (5′-TAAATGAGTTGAGCCACG-3′) and O-EN23 (5′-CTC CATCGCTGTCCAACCG-3′); and O-PEN115M13R1F1 (5′-GTAAATACAT GAATGAGC-3′) and O-EN14 (5′-CCAAAATACTCCATTGCGAG-3′). These three primer pairs spanned the regions between *luxR1* and *luxC*, *luxE* and *luxR2*, and *luxR2* and *ribG*, respectively.

Genetic complementation of the aldehyde deficiency. A derivative of *V. salmonicida* NCMB 2262^{T} that harbored pEN135, which constitutively expressed the *V. fischeri* ES114 aliphatic-aldehyde synthesis (AAS) genes, *luxCDE*, was constructed. To construct the plasmid pEN135, the *V. fischeri* *lux* gene region containing *luxRICDBEG* (*luxA* was previously deleted) was excised from pKV17 (45) by using SalI and ligated into the *V. fischeri* cloning vector pVO8 (47). The *luxR* and *luxI* genes were removed from the resulting plasmid by KpnI and BglII double digestion, followed by ligation of the digest with a double-stranded oligonucleotide linker that contained KpnI- and BglII-complementing ends; a SalI site was in the middle of the linker. *E. coli* DH5α was transformed with the resulting product, and transformants were selected on LB-Cam medium. A plasmid that carried the *V. fischeri* *luxCDBEG* genes under the control of the *lacZ* promoter was isolated (see Fig. 2A). The in vitro EZ::TN <KAN-2> insertion kit (Epicenter Technologies Inc., Madison, WI) was used to create a null insertional mutation located 302 bp into the *luxG* ORF, resulting in pEN135. The pEN135 plasmid was mated into *V. salmonicida* in a triparental mating as described above.

Measurement of bacterial culture luminescence. Luminescence was measured with a TD-20/20 luminometer (Turner Designs Inc., Sunnyvale, CA). The luminescence of late-log-phase cultures (OD, 0.7 to 0.9) at the time of the assay both with and without the addition of decyl aldehyde (4.5-µg/ml final concentration) was measured.

Total RNA isolation. *V. salmonicida* was streaked onto SWT agar and incubated for 4 days at 16°C. An isolated colony was inoculated into 10 ml of SWT broth and grown with shaking at 16°C to an OD of 0.5. Two milliliters of broth (containing about 2×10^8 cells) was pelleted, and total RNA was extracted from the pelleted cells by using the RNeasy kit (QIAGEN, Valencia, CA). The resulting total RNA fraction (about 1 µg) was mixed with 2 µg of DNase I in a 75-µl reaction mixture containing 30 mM Tris-HCl (pH 7.8), 50 mM NaCl, and 10 mM MgCl$_2$ in nuclease-free water. The reaction mixture was incubated at 37°C for 1 h to digest any contaminating DNA. Following this incubation, total RNA was reisolated using the RNeasy protocol and quantified using a Biophotometer (Eppendorf, Hamburg, Germany). PCR was used to confirm that DNase I had removed DNA from the sample.

cDNA synthesis and reverse transcriptase PCR (RT-PCR) analysis. The expression of *lux* genes was determined using the SuperScript II RNase H$^-$ reverse transcriptase kit (Invitrogen, Carlsbad, CA). The following components were combined in a first-strand cDNA synthesis reaction mixture: 2 pmol of sense or antisense gene-specific primers, 1 to 1,000 ng of total RNA, and each deoxynucleoside triphosphate to a concentration of 10 mM in a final volume of 12 µl. The cDNA was synthesized according to the manufacturer's protocol. For second-strand PCR, 1 µl of the resulting cDNA mixture was combined with 10× PCR buffer (200 mM Tris-HCl [pH 8.4] and 500 mM KCl), 2 mM MgCl$_2$,

deoxynucleoside triphosphates (250 µM [each]), gene-specific primers (10 µM [each]), and *Taq* polymerase to a final volume of 50 µl. The mixture was subjected to the following amplification conditions: 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 30 cycles, followed by a one-time final extension step at 72°C for 7 min. Gene-specific primers were as follows: *luxE* sense primer (CPO7), 5′-ATTTATGAGTACGCCACAAG-3′; *luxE* antisense primer (CPO8), 5′-GGTA CTCGCTTTCTTTGAAA-3′; *luxR2* sense primer (CPO11), 5′-ATATAACGG GTTCATTGCTC-3′; *luxR2* antisense primer (CPO12), 5′-TGCCTACAAGAA CTAACCAA-3′.

Preexperiment passage of *V. salmonicida* strains in fish and dosage titration. An animal model was developed that controlled for variability due to water conditions (temperature and osmolarity), equitable feeding, and fish stock. The protocol was approved by the institutional review board for the ethical treatment of animals at the Norwegian School of Veterinary Science. Atlantic salmon fry used for the challenge averaged 50 g (wet weight) and were presmolts. Salmon were kept in 200-liter freshwater tanks at 6 to 7°C with standard oxygenation and a flow rate of 50 liters/h. Both the *luxA* mutant EN4 and the wild type NCMB 2262 were separately passaged for 2 days in Atlantic salmon fry to confirm that the strains had not lost pathogenicity during in vitro culture (49). Mutant and wild-type clones were recovered from their hosts by inserting a sterile probe into the head kidneys and streaking for single colonies. These passaged strains were tested in dosage titration experiments to find the optimum infective doses. A series of twofold dilutions of these fish-passaged strains (1×10^8 to 5×10^6 CFU per fish) was injected into the prepelvic abdominal regions of the fry. Mortality was monitored each day over a 25-day time course.

Single-strain challenge infection of Atlantic salmon. Fish were injected in the abdominal cavity with 1×10^8 CFU of the wild type or the *luxA* mutant grown overnight in SWT broth. Fish were fin tagged according to the inoculum received and placed in a single tank. The tank was observed twice daily for dead fish. *V. salmonicida* does not survive in freshwater and does not transfer among individuals kept in the same tank (8, 39; unpublished data). Mortality was tracked over 1 month, and *V. salmonicida* cells were recovered from the head kidney of each dead fish by plating homogenates onto SWT agar. The identity of the infecting strain (wild type or mutant) was confirmed by sequential plating on SWT-Kan and then SWT agar plates.

Competition between *luxA* mutant and wild-type *V. salmonicida* strains infecting Atlantic salmon. The fish-passaged *luxA* mutant EN4 and its wild-type parent strain were grown overnight in SWT. The two strains were combined in an approximately 1:1 ratio, and a total of 1.1×10^8 CFU per fish was injected into the abdominal cavities of 50 fish. Fish were fin tagged accordingly and placed in a single tank. The tank was observed twice daily for diseased and dead fish. Tissue from the head kidney of each dead fish was first streaked onto nonselective SWT plates for single colonies. Subsequently, colonies were patched onto SWT-Kan and SWT, and their differential growth was used to calculate the ratio of the mutant cells to the wild-type cells in the infected fish. A similar procedure was used in an in vitro competition experiment performed with SWT broth. Cultures were inoculated to an OD of 0.01 and grown to stationary phase.

Nucleotide sequence accession number. The nucleotide sequence of the *V. salmonicida* *lux* gene cluster has been submitted to GenBank (accession no. AF452135).

RESULTS

The structural *lux* genes are conserved in *V. salmonicida*. The order of the structural genes of the *V. salmonicida* *lux* operon is *luxCDABEG*, which agrees with the gene orientation in *lux* operons from other luminous bacterial species (Fig. 1). The amino acid sequences corresponding to these six genes from *V. salmonicida* and *V. fischeri* MJ1 are also highly conserved, with levels of identity ranging between 65 and 87%
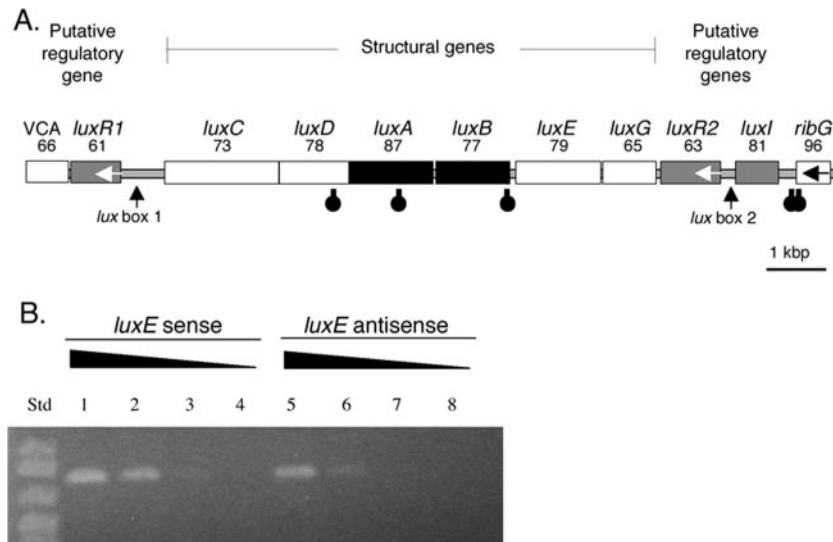
FIG. 2. Bioinformatic and RT-PCR transcriptional analysis of the *V. salmonicida lux* region motifs. (A) The putative direction of transcription is from left to right unless indicated otherwise by an arrow. Black and gray highlighting denotes ORFs corresponding to luciferase genes and *lux* regulatory genes, respectively. The number listed below each gene name is the percentage of identity between the predicted amino acid sequence for the ORF and the sequence of the equivalent *lux* protein in *V. fischeri* strain MJ1. Stem-loop symbols below the ORFs represent the approximate locations of putative *rho*-independent transcriptional terminators. VCA is a homolog of the gene for the VCA0181 protein, a *V. cholerae* hypothetical protein with homologs found in both *V. salmonicida* and *V. fischeri*. (B) RT-PCR products of sense (lanes 1 to 4) and antisense (lanes 5 to 8) transcripts of *luxE* amplified from *V. salmonicida* total cellular RNA. The RNA extract was either undiluted (lanes 1 and 5), diluted 1:10 (lanes 2 and 6), diluted 1:100 (lanes 3 and 7), or diluted 1:1,000 (lanes 4 and 8). RT-PCR performed with no added RNA gave no product (data not shown). In lanes 1, 2, 5, and 6, a band matching the predicted 803-bp *luxE* product can be seen. Std, standard size markers; from top: 1,000, 900, 700, and 500 bp.

(Fig. 2A). In addition, there are no detectable deletions or insertions in this six-gene locus.

The *V. salmonicida luxR* and *luxI* homologs are arranged differently from those in the *V. fischeri lux* region. In contrast to the conserved arrangement of the *lux* structural genes, homologs of the quorum-sensing regulatory genes *luxR* and *luxI* have a novel arrangement in *V. salmonicida* (Fig. 1) compared to *V. fischeri* (1, 17). There are two copies of the *luxR* homolog, one located upstream and one downstream of the structural gene cluster. These homologs were designated *luxR1* and *luxR2*, and their predicted protein sequences showed 61 and 63% amino acid identity to the *V. fischeri* MJ1 *luxR* gene product, respectively (Fig. 2A). The *luxR1* and *luxR2* predicted protein sequences showed only 61% amino acid identity to each other, suggesting that there has been considerable divergence since the apparent gene duplication event. Like the *luxR* gene of *V. fischeri*, both *luxR1* and *luxR2* of *V. salmonicida* appear to be transcribed in a direction opposite to that of the structural *lux* genes. In addition, there is a *luxI* homolog adjacent to, but apparently divergent from, the *luxR2* homolog (Fig. 2A). The product of this *V. salmonicida luxI* homolog showed 81% amino acid identity with the product of *V. fischeri* MJ1 *luxI*. Between the *luxR1* and *luxC V. salmonicida* ORFs, the position where *luxI* is located in *V. fischeri*, are 559 bp of sequence that do not contain any apparent ORFs (Fig. 2A).

The arrangement of the *V. salmonicida lux* cluster is conserved among different strains. PCR was used to determine whether the *lux* gene arrangement present in *V. salmonicida* strain NCMB 2262[T] was found in four other strains of this species. The three sets of PCRs (see Materials and Methods) covering the *luxR1-luxC*, *luxE-luxR2*, and *luxR2-ribG* regions all

produced products of the predicted 1.6-kbp, 1.9-kbp, and 1.9-kbp lengths, respectively, for each of the five *V. salmonicida* strains (data not shown). These results suggest that the arrangement of the *lux* gene cluster in the regions amplified is conserved within *V. salmonicida*.

The aliphatic-aldehyde deficiency can be genetically complemented. One explanation for the aliphatic-aldehyde deficiency and the reduced luminescence of *V. salmonicida* is that the metabolism of this species is unable to provide the substrate(s) required for the synthesis of aliphatic aldehyde. To examine this hypothesis, *V. fischeri* AAS genes were expressed in *trans* in *V. salmonicida* and the resulting luminescence per cell was determined. *V. salmonicida*, expressing in *trans* the AAS gene-carrying plasmid pEN135 (Fig. 3A), was detectably luminous and produced at least 1,200-fold more luminescence than the wild-type strain (Fig. 3B). The addition of exogenous aliphatic aldehyde did not result in a significant increase in luminescence in *V. salmonicida* cells harboring pEN135 (Fig. 3B). These data suggest that *V. salmonicida* is not physiologically limited in its ability to produce the substrates required for aliphatic-aldehyde synthesis. In addition, because *V. salmonicida* cells harboring pEN135 and those harboring the vector plasmid pV08 have the same growth rate (3.3 h per generation), the production of additional aliphatic aldehyde does not appear to be toxic to *V. salmonicida*. It is unlikely that the collateral presence of a copy of the *V. fischeri luxB* gene in the construct (Fig. 3A) is responsible for the enhanced luminescence expression observed because aldehyde addition alone increased the luminescence of wild-type cells by several orders of magnitude (Fig. 3B).
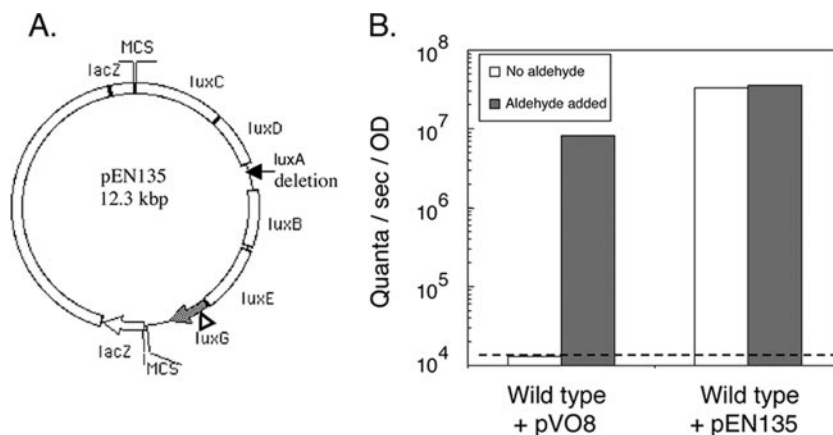
FIG. 3. Genetic complementation of the aliphatic-aldehyde deficiency in wild-type *V. salmonicida*. (A) The plasmid used for complementation studies, pEN135, contains the *V. fischeri* ES114 AAS genes, *luxCDE*, as well as the luciferase subunit gene *luxB*. The other luciferase subunit gene, *luxA*, is deleted, and *luxG* (gray) is inactivated by a transposon insertion (triangle). MCS, multiple cloning site. (B) Comparison of luminescence produced by wild-type *V. salmonicida* harboring the vector plasmid pVO8 and that produced by wild-type *V. salmonicida* harboring the AAS gene-carrying plasmid pEN135. Aliquots of cultures (late exponential phase of growth; OD at 600 nm, 0.7 to 0.9) were either immediately measured photometrically or supplemented with aliphatic aldehyde before measurement. The dashed line represents the limit of photometric detection. Data shown are representative of the results of three independent experiments.

The intergenic regions of *V. salmonicida* lux genes share conserved elements with similar regions in *V. fischeri*. The intergenic regions between *luxR1* and *luxC* and between *luxR2* and *luxI* were analyzed for possible transcriptional promoter elements. The putative transcriptional initiation loci upstream of both *luxC* and *luxI* in *V. salmonicida* contain substantial similarities to the region upstream of *luxI* in *V. fischeri* MJ1 (Fig. 4). There are two putative regulatory elements, *lux* box 1 (between *luxR1* and *luxC*) and *lux* box 2 (between *luxR2* and *luxI*), in *V. salmonicida*. Both of these elements precede a −10 region that is identical to that found in *V. fischeri* MJ1 (Fig. 4). In addition, *V. salmonicida* lux box 1, *V. salmonicida* lux box 2, and the *V. fischeri* lux box are centered at −42.5, −43.5, and −42.5 bp, respectively, upstream of their predicted transcriptional start sites (6) (Fig. 4). Putative ribosomal binding sites

and start codon loci, determined by sequence similarities, are conserved between *V. fischeri* MJ1 and *V. salmonicida* for *luxI*, *luxC*, *luxA*, *luxB*, and *luxG* (Fig. 4 and data not shown). However, unlike *V. fischeri* MJ1, *V. salmonicida* has no apparent start codon at the 5′ end of the *luxD* ORF.

Stem-loop structures in the *V. salmonicida* lux region are conserved. To assess how transcription of the *V. salmonicida* lux operon may be terminated, the sequence data were screened for stem-loop structures that may function as *rho*-independent transcriptional-termination factors. We identified three putative stem-loop structures in the *lux* gene cluster of *V. salmonicida*. These structures are located in the middle of the *luxD* and *luxA* coding regions and at the 3′ end of *luxB* (Fig. 2A). The sequence of the stem-loop at the *luxB* terminus in *V. salmonicida* (AAAAGAATGACAGAATTA
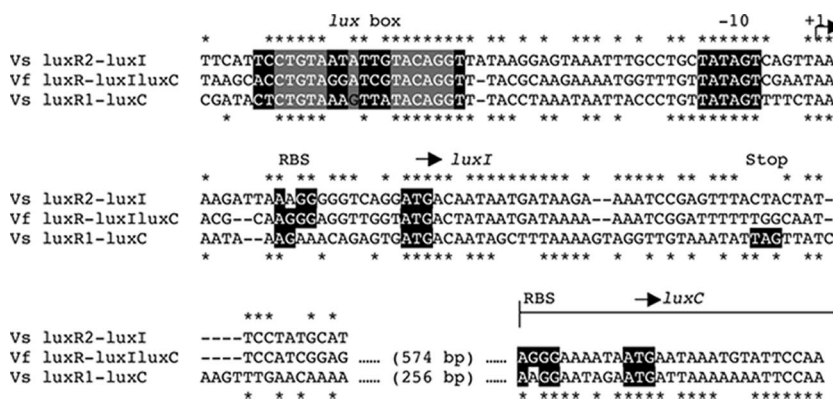

FIG. 4. Sequence comparison of the intergenic regions between *luxR2* and *luxI* of *V. salmonicida* (Vs luxR2-luxI), *luxR* and *luxIC* of *V. fischeri* MJ1 (Vf luxR-luxIluxC), and *luxR1* and *luxC* of *V. salmonicida* (Vs luxR1-luxC). The *V. fischeri* luxR-luxIC and *V. salmonicida* luxR1-luxC sequences have been extended into their *luxC* ORFs. Identical nucleotides in the *V. salmonicida* luxR2-luxI and *V. fischeri* luxR-luxIC sequences are indicated with asterisks above the alignment. Identical nucleotides in the *V. fischeri* luxR-luxIC and *V. salmonicida* luxR1-luxC sequences are indicated with asterisks below the alignment. The highlighted sequences are based on motifs conserved around *V. fischeri* lux genes (17). These regions include a *lux* box, a −10 promoter region, and known or putative ribosome binding sites (RBS). Start (ATG) and stop (TAG) codons are also highlighted. Gray boxes highlight sequences required for a functional *lux* box in *V. fischeri* MJ1 (6).

ACTCTGCCATTCTTTT) is similar to those in other luminous bacteria but was predicted to have greater thermostability ($\Delta G = -15$ kcal/mol) than the equivalent structures in *P. leiognathi* (-12 kcal/mol), *V. harveyi* (-10 kcal/mol), and *V. fischeri* MJ1 (-5 kcal/mol). In *V. salmonicida*, there are two additional predicted stem-loops, between *luxI* and *ribG* (Fig. 2A). It was previously shown by reporter gene analysis that an equivalent stem-loop in *V. fischeri*, also located between the *lux* operon and *ribG*, is a bidirectional transcriptional terminator (42).

Sense and antisense transcripts of *luxE* and *luxR2* are produced by *V. salmonicida*. The organization of the *V. salmonicida lux* operon, coupled with its aldehyde-deficient luminescence physiology (13), suggested that a long transcript from the rightward *luxR2* promoter (Fig. 2A) might exert antisense control over the expression of *luxE*, one of the genes required for aldehyde synthesis. In support of this hypothesis, when total RNA isolated from growing cells of *V. salmonicida* was mixed with primers to amplify either sense or antisense *luxE* or *luxR2* transcripts by reverse transcriptase PCR, we detected sense and antisense transcripts of both genes (Fig. 2B; *luxR2* data not shown). The relative amounts of the sense and antisense *luxE* products resulting from the semiquantitative RT-PCR suggested that the level of the sense transcripts was higher than that of the antisense transcripts.

Complementation of the *luxA* mutation. Complementation of the *luxA* mutation in vitro was observed in the *luxA* mutant EN4 harboring the plasmid pEN134 compared to EN4 harboring the vector parent plasmid pV08. Specifically, in the presence of decyl aldehyde (10 μl/ml), EN4 harboring the plasmid pEN134 or pV08 produced 10^9 or $<10^4$ quanta/s/OD unit, respectively. In the absence of added decanal, an aliphatic aldehyde, EN4 harboring either pEN134 or pV08 produced 5×10^5 or $<10^4$ quanta/s/OD unit, respectively.

Atlantic salmon infected with a *luxA* mutant show delayed mortality. Dose titration data showed that inoculation with approximately 1×10^8 CFU of *V. salmonicida* produced a consistent level of mortality in salmon such that the criterion for a 50% lethal dose would be observed by approximately day 10 (data not shown). This dose is at the upper range of the 50% lethal doses determined previously (5×10^6 to 1×10^8 CFU) by Wiik et al. (49), perhaps because the fry we used were particularly robust. In the large-scale studies, each fish was injected with 1×10^8 CFU of either the *luxA* mutant (EN4) or its wild-type parent. Mortality was observed starting at day 2 and continued until day 25 (Fig. 5A). There was no evidence of cross contamination between infected animals: the postmortem examination of fish injected with wild-type cells did not reveal the presence of the mutant strain; the converse was also true. Dead fish did not display pathology on their external surfaces; however, their livers were atypically fatty, and points of hemorrhaging were observed in both the intestines and the kidneys. Levels of mortality in the two branches of the experiment were identical until day 9 (Fig. 5A), at which time the mutant showed a dramatic delay in its ability to kill fish compared to the wild type. The difference between the survival plots for the mutant and the wild type was statistically significant (log rank test, $P = 0.0002$; Wilcoxon test, $P = 0.0015$).

The *luxA* mutant is outcompeted by the wild type during infection. Cells of the *luxA* mutant EN4 and its wild-type par-
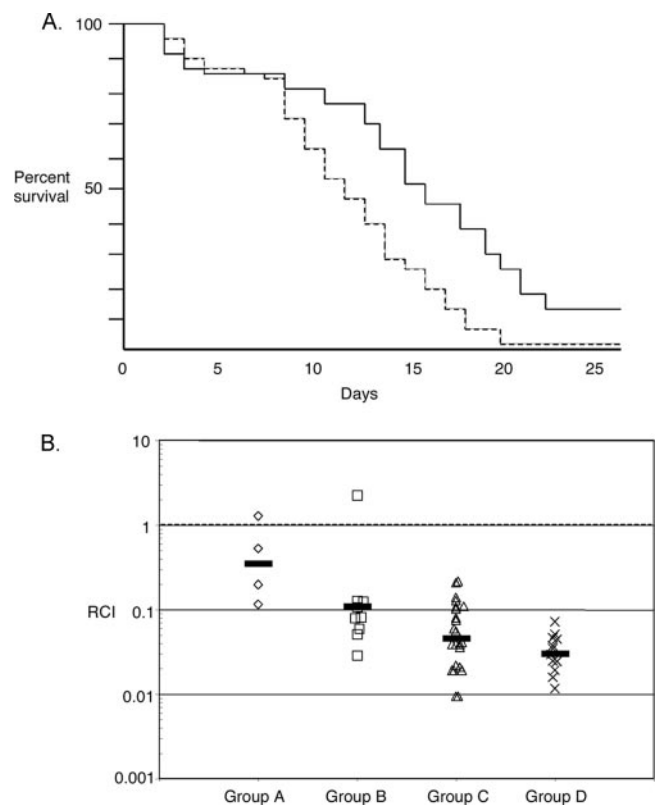

FIG. 5. Data from fish virulence experiments comparing the *luxA* mutant EN4 and its wild-type parent. (A) Survival plot for Atlantic salmon fry infected with either the *luxA* mutant EN4 (solid line) or the wild type (dashed line). Mortality among the fish infected with the mutant was significantly delayed compared to that among the fish infected with the wild type (log rank test, $P = 0.0002$; Wilcoxon test, $P = 0.0015$). Inputs were 9.7×10^7 and 1×10^8 CFU per fish for the mutant and the wild type, respectively. (B) Competition experiment with the *luxA* mutant EN4 and the wild type. Fish were grouped according to the duration of infection before death (A, 1 to 5 days; B, 6 to 10 days; C, 11 to 15 days; and D, 16 to 20 days). The relative competitive index (RCI) of the *luxA* mutant for each fish was determined by dividing the output ratio for the two strains (mutant cells to wild-type cells) in the head kidney of the dead fish by the input ratio in the mixed inoculum. An RCI of less than 1.0 indicates that the wild type outcompetes the mutant. Each symbol on the graph represents the RCI calculated for one fish, and the geometric mean for each group is indicated by a bar.

ent were combined in approximately equal numbers (1.07:1), and a portion of this mixed inoculum was injected into the abdominal cavities of 50 fish. The final ratio of mutant cells to wild-type cells in the infection was assessed for each fish that died over the 25-day experiment. Mortality groupings were made according to the duration of infection as a method of identifying stages of the disease (group A, 1 to 5 days; group B, 6 to 10 days; group C, 11 to 15 days; and group D, 16 to 20 days). The mutant was attenuated approximately threefold during the first day postinoculation, and the competitive disadvantage continued throughout the experiment, with the competitive index decreasing from 0.35 to 0.11 to 0.05 to 0.03 for groups A to D, respectively (Fig. 5B). To determine whether there was a significant growth rate difference between the strains during growth in culture, mixed 1:1 inocula of mu-

tant and wild-type cells (OD, 0.01) were added to SWT broth and replicate cultures were grown overnight. The ratios of mutant to wild-type cells at stationary phase ranged from 0.85 to 0.96.

DISCUSSION

We have characterized the luminescence gene cluster of *V. salmonicida* with the goal of understanding the mechanism and role of this organism's cryptic bioluminescence. To this end, we discovered a unique gene structure that includes an unusual arrangement of quorum-sensing genes. We also showed that a mutation in *luxA* could attenuate *V. salmonicida* pathogenesis. Although the nature of the association between *lux* gene expression and virulence remains unknown, the implications of uncovering a new class of virulence factors are significant. The introduction and development of *V. salmonicida* genetics now permits future investigators to explore virulence in what perhaps is the only truly natural vertebrate model system for studying pathogenesis in the genus *Vibrio*.

The prevalence of luciferase genes in *Vibrionaceae* species that do not produce detectable light has led to the question of what role, if any, there is for the activities encoded by these genes in nonluminescent bacteria. In at least some of these bacteria, the luciferase genes are expressed but the reaction catalyzed by their corresponding proteins is limited by the availability of the aliphatic-aldehyde substrate. The experimental addition of an aliphatic aldehyde results in detectable light emission from these cells (12, 13; unpublished results). Such cryptic luminescence in *V. salmonicida* has been described previously and may occur as a result of several possible explanations: (i) one (or more) of the genes encoding AAS enzymes is absent or nonfunctional; (ii) all the enzymes are synthesized, but the cell produces insufficient substrates for the AAS reaction; and/or (iii) the relative level of expression of the AAS genes is reduced.

The *V. salmonicida lux* operon contains all of the structural *lux* genes that are required for bioluminescence (Fig. 1). These genes, *luxCDABE*, are organized in the typical arrangement observed in other known *lux* operons. The *V. salmonicida lux* nucleotide sequences are most similar to those of its close relative, *V. fischeri*, and there are no detectable deletions or insertions in the structural *lux* genes. These data suggest that the aliphatic-aldehyde deficiency is not caused by the absence of the AAS genes.

We also determined that the synthesis of aliphatic aldehyde is not limited by the ability to provide substrates for AAS (Fig. 3B). The precursors for aliphatic aldehyde, namely, saturated long-chain fatty acids and reducing equivalents (5, 43, 48), are apparently readily available for AAS in *V. salmonicida*. Therefore, we explored the hypothesis that reduced or aberrant expression of the AAS genes may explain cryptic bioluminescence in *V. salmonicida*.

The transcription of bioluminescence genes is complex but has been well studied with other models. For example, the 218-bp intergenic region between the start codons for *luxR* and *luxI* in *V. fischeri* is the site where the transcription factors LitR (12) and LuxR (41) bind to divergently promote the transcription of *luxR* and *luxI*, respectively. Although the exact binding site for LitR is not known, LuxR, in complex with an acyl-homoserine lactone autoinducer, binds at a specific region called the *lux* box (7). The intergenic region between *luxR1* and *luxC* in *V. salmonicida* also contains a conserved *lux* box and a −10 region that is identical to that of *V. fischeri*. In addition, there is a similar translational initiation region for a *luxI* gene (Fig. 4); however, there is no *luxI* homolog following this locus in *V. salmonicida*. Instead, the region between *lux* box 1 and *luxC* in *V. salmonicida* consists of only half the number of nucleotides found between the *lux* box and *luxC* in *V. fischeri*. We also detected evidence for a 2-bp frameshift that may result in a premature stop codon (TAG) (Fig. 4). Following this intergenic region in *V. salmonicida*, elements of the translational promoter for *luxC* are conserved between *V. salmonicida* and *V. fischeri*. Thus, the presence of the conserved transcriptional initiation elements suggests that the transcription of the *V. salmonicida lux* operon may be initiated in a fashion similar to that of the *V. fischeri* operon but that the first gene transcribed in *V. salmonicida* is the *luxC* homolog and not a *luxI* homolog. Strong stem-loop structures are predicted in the *V. salmonicida lux* gene cluster that are shared across bioluminescent taxa. The role that these stem-loops play in transcriptional modification remains unknown. Additional findings that *V. salmonicida* produces an as yet undescribed autoinducer (13; data not shown) and encodes a homolog of the *V. fischeri* luminescence regulatory gene *litR* (12; data not shown) suggest that *V. fischeri* and *V. salmonicida* share other genetic control mechanisms for bioluminescence expression. However, the natures of the expression differ.

Homologs of the *luxR* and *luxI* regulatory genes in *V. salmonicida* have a novel arrangement. First, there are *luxR* homologs located both upstream and downstream of the *V. salmonicida* structural *lux* genes (Fig. 1). Second, there is a *luxI* homolog downstream of *luxR2*. While this arrangement of *luxR::luxI* is found in *V. fischeri* as well, the location of the regulatory gene pair downstream rather than upstream of the structure genes is unique to *V. salmonicida*. In addition, the bidirectional transcriptional terminator at the end of *luxG* in other bioluminescent bacteria is decoupled from *luxG* in *V. salmonicida* but remains upstream of *ribG*. This novel genetic structure suggests a transcriptional model in which rightward-sense transcription from *luxC* to *luxE* may continue, generating antisense *luxR2*. Conversely, leftward-sense transcription from *luxR2* may produce antisense *luxE* transcripts. RT-PCR detected both sense and antisense transcripts for *luxE* and *luxR2*. These data support the model that antisense gene regulation drives cryptic bioluminescence by reducing the expression of *luxE* and, therefore, AAS. Preliminary data suggest that a mutation in *luxR2* delays the onset of peak bioluminescence but eventually produces a higher peak level than that for the wild type (data not shown). Further mutational and quantitative PCR analyses of each *lux* gene will be needed to test this model in which antisense RNA contributes to the cryptic bioluminescence phenotype of *V. salmonicida*.

Given the novelty of the arrangement of the luminescence gene cluster in *V. salmonicida*, is cryptic bioluminescence merely a remnant of an ancestral phenotype, or does it serve a current biological function? All five *V. salmonicida* strains tested appear to share the same *lux* gene cluster organization. The conservation of the arrangement within the species suggests that a function of the *lux* gene cluster may exist. To begin

answering questions of functionality, we asked whether an insertional mutation in *luxA* would affect the virulence of *V. salmonicida*.

In a single-strain challenge experiment, the mutagenesis of *luxA* resulted in a marked delay in mortality among *V. salmonicida*-infected Atlantic salmon compared to that induced by the wild type. Similarly, in a mixed-strain competition experiment with the mutant and the wild type, the *luxA* mutant was attenuated 3- to 50-fold depending on the duration of infection. These data demonstrate that the disruption of *luxA* attenuates *V. salmonicida* colonization. However, the mechanism underlying this attenuation is not known. We hypothesize that the attenuation may be directly due to the loss of luciferase, which results in the elimination of the dark luciferase reaction. The dark luciferase reaction produces toxic oxygen radicals that may serve as a direct virulence agent or a stimulant for bacterial DNA repair (15, 22, 23, 25). Alternatively, the luciferase reaction may function as an alternative pathway to provide oxidized flavin at low oxygen tensions which may aid colonization if oxygen becomes limited (4, 26).

In the symbiosis of *V. fischeri* with the Hawaiian squid *Euprymna scolopes* (3), bioluminescence has been shown to be a colonization factor for the bacterium (46). At the cellular level, the luciferase reaction of *V. fischeri* is associated with symbiosis-induced host development including epithelial-cell swelling, as well as with the maintenance of persistent colonization of the host squid tissue (46). Thus, a similar, but nonbeneficial, role in pathophysiology may underlie the attenuation observed in the *luxA* mutant of *V. salmonicida*. In summary, the development of molecular tools for *V. salmonicida*, together with the creation of a natural salmon infection model, have led to a new system with which to evaluate the genetic structure, function, and evolution of bacterial bioluminescence in pathogenesis.

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